Saskatchewan
Agriculture
and Food

FINAL REPORT

Agriculture Research Branch
Saskatchewan Agriculture and Food

20000331

AMERICAN FOULBROOD (AFB) DISEASE

Funded by
Technology Adoption Demonstration

March 2002
Prepared by: Saskatchewan Beekeepers Association

102-05775/

A STUDY TO DETERMINE IF HONEY SAMPLES CAN BE USED TO DETECT AMERICAN FOULBROOD DISEASE IN HONEY BEE COLONIES

Project #20000331

Submitted by:

Saskatchewan Beekeepers Association

April 2002

SUMMARY

American Foulbrood is a disease of honeybee colonies caused by a bacterium, *Paenibacillus larvae*. The disease is contagious and can spread from colony to colony and spread quickly within an industry. Recently, a new strain of the bacterium has been discovered which is resistant to the only current registered antibiotic control.

Research in other parts of the world has indicated that honey analysis can be used to determine the presence of American Foulbrood spores within the honey even before a colony shows visible signs of infestation. The purpose of this study was to determine if honey samples could be used to determine the presence of American Foulbrood. Secondarily, inspections of live colonies were preformed to look for colonies infected with American Foulbrood disease. Any infections of American Foulbrood that were discovered and isolated would then be tested for resistance to the antibiotic, oxytetracycline.

The honey sampling indicated that the causative organism of American Foulbrood could be found in honey samples. However, the laboratory analysis was difficult and extremely complicated and it became evident that the examination protocol was not able to eliminate other bacteria. Consequently, many of the samples were contaminated with other bacteria and the samples that were thought to be demonstrating resistance were suspect because of the presence of contaminating bacteria.

At the end of the study the 46 samples that showed any resistance to the antibiotic were sent to the Agriculture Canada Research Station at Beaverlodge for further testing. They were able to obtain pure cultures and, when tested, all of these showed a high susceptibility to oxytetracycline.

The current study indicates that the methodology is not yet refined to the point where this could be used as a survey technique with absolute certainty of the results. The field survey indicated that there was a significant amount of American Foulbrood present and that there is a need to increase the survey efforts annually. Once the technique is refined this may be a plausible survey technique. Thirdly, it can be concluded that the resistant form of American Foulbrood has not yet become established in Saskatchewan.

The isolation of American foulbrood, *Paenibacillus larvae* subsp. *larvae*, from random honey and honeycomb samples in Saskatchewan, 2001

Methods

Each honey sample is weighed to 5 g and transferred into a covered culture tube. The specimens are heat shocked in an 80-82°C water bath for 15 minutes. The honey is then directly inoculated onto 3 MYPGP agar plates, one BHIT plate and one BHIT plate containing Oxytetracycline (BHITwO). The plates are incubated at 36°C. The MYPGP plates are subjected to 5 percent carbon dioxide and the BHIT plates are kept under aerobic conditions. The numbers of colonies are counted after days -one, -three, -seven and -fourteen.

After day-fourteen, the colonies are examined and compared to the positive control. Each culture being suspicious of containing *P. larvae* is tested for: 1) a catalase reaction using 3 percent Hydrogen peroxide and, 2) a Gram reaction using a general Gram staining technique. The cultures containing Gram-positive, spore-forming rods are further examined and are sub-cultured onto BHIT containing Nalidixic acid (BHIT+N) to decrease contamination of other species. Once a pure culture is obtained, the BiOLOG MicroLog™ Microbial Identification System is utilised as an identification confirmation tool.

The honeycomb samples are treated similarly. Honeybee larval scales are removed from individual cells and added to 5 g of sterile honey. The specimens are heat shocked, inoculated and incubated similarly to the honey samples. There were 139 honey samples and 32 honeycomb samples tested, equalling a total of 171 samples tested.

Results

Please see the attached spreadsheets: 1) a list of results of the American foulbrood screening project and 2) pie graphs depicting the results. **(* Please note that the results may not be absolute due to complications that arose throughout the project. A further detailed hard copy of the results is also available which includes information about the catalase testing, Gram-staining, colony enumeration and morphology, bacterial cell and spore morphological descriptions and retested samples.)**

Bacterial growth was evident on the majority of the agar plates. Many of the plates contained a variety of cultures, some of which included *P. larvae*. Please see the list below that summarizes the complications that arose throughout the project:

Complications:

- After 2 weeks of incubation agar plates frequently desiccate, regardless of how thick the media was poured into the petri dishes; BHIT tends to dry out more quickly than MYPGP, therefore the MYPGP plates may have to be relied upon for analysis after the 2 week period, unless fresh BHIT cultures are maintained
- After heat-shocking, many other *Bacillus* spp./spore-forming bacteria contaminate the plates making it difficult to ID *P. larvae* rods/spores
- It would be extremely beneficial to have a more selective media for *P. larvae*, such as the media to select for *B. cereus*, for example
- *P. larvae* vegetative cells (rods) and spores (oval-shaped) can be easily confused with other *Bacillus* rods and spores, i.e., it is very difficult to distinguish between *Bacillus* spp. with a compound microscope, i.e., one would almost require an electron microscope for morphology comparison
- There is a significant chance of identifying a 'false-negative' sample, possibly due to an uneven distribution of spores in the honey samples or lack of growth on the agar plates for unknown reasons
- Often when a plate is contaminated, it is very difficult to isolate *P. larvae* due to the other faster-growing, contaminating species present which predominate and swarm the agar plates
- Various serial dilution methods were attempted but unsuccessfully rendered *P. larvae* colonization from the swarming, contaminating species

- Subculturing on BHIT+N only *occasionally* results in the isolation of *P. larvae* from other species
- Nalidixic acid added to the media did not appear to knock out contaminating species of bacteria (perhaps *P. alvei* though)
- BiOLOG cannot be utilized unless a pure culture is attained, and unfortunately, a pure culture is often difficult to attain
- The agar plates often had a fair amount of condensation in them, causing water droplets to settle on the surface and prohibit individual colony development, i.e., often smears where obtained rather than individual, pure colonies (uncommon for *P. larvae* under normal conditions); a water-pour-off method before inoculation occasionally helped
- The positive IDs required OTC resistance retesting because often contaminating species were resistant to OTC and approached the disk but *P. larvae* was not resistant and did not approach the disk (it was difficult to determine each case by simply viewing the BHITwO plates); sometimes *P. larvae* could not be isolated from the contaminating species, rendering an inconclusive result with respect to OTC resistance
- The catalase test appeared positive for some positive *P. larvae* samples due to contamination, i.e., other catalase positive bacteria were present in the culture
- Colonies of *P. larvae* have various appearances including: individual, round, opaque colonies of various sizes (0.5 mm to 5 mm in diameter), smears, and individual colonization overlying a contaminating smear
- As well, the vegetative cells (rods) have a variety of morphological features: long and narrow, short and stout, as spores, or as a developing cell into a spore
- Two days after inoculation, the vegetative cells can start developing into spores which can create difficulty in identification
- Often a smear (contaminating species) will predominate on an agar plate and small, individual colonies (possibly *P. larvae*) will develop on the surface of the smear; the colonies are difficult if not impossible to isolate from the smear
- ***P. alvei* is not included in the BiOLOG database; instead, it is identified as *P. larvae* ss. larvae, i.e., destroying the confirmation tool of positive IDs! A new method of confirmation will have to be utilized, such as: 1) PCR analysis, 2) fatty acid analysis, 3) sending the "positive" samples to the USDA lab to be tested, 4) electrophoresis and DNA analysis**
- We do not have the most accurate BiOLOG plate reading capability, i.e., we are required to visually read each well as "-/+" which may cause discrepancy. There is a tool (the MicroStation™ reader) that can be utilized to perform automated readings of the various carbon sources that have been metabolized in each well
- Often the Gram stains will appear red and purple; rather than those samples being Gram-negative, they are often Gram-positive samples that are contaminated with Gram-negative species
- On occasion, the BiOLOG system was unsuccessful with providing an ID. This could be due to: 1) an inadequate database, 2) an impure culture, 3) perhaps the number of times the cultures were subcultured prior to testing

(often the cultures required 2-5 subcultures before a pure culture was obtained), or 4) possible error in testing

Discussion and Suggestions

Overall, the honey screening protocol successfully allows for *Paenibacillus larvae* to be isolated from honey and honeycomb samples and resistance to Oxytetracycline can be determined. However, a confirmation tool is required to obtain confident results until more research is conducted on honey testing for American foulbrood. Such tools might include other biochemical tests, fatty acid analysis, PCR analysis or other DNA analytical methods such as electrophoresis. It would be extremely beneficial to develop a media selective for *P. larvae* ss. *larvae* for isolation from contaminants including other spore-forming *Bacillus* spp. Each sample may require an increased number of inoculations- some samples were "hit and miss," i.e., *P. larvae* was occasionally isolated in only a second sample trial for reasons unknown.

Approximately twenty-eight percent of the honey samples tested was found to contain *P. larvae*. Of the 46 positively tested samples, only 2 showed absolute susceptibility to Oxytetracycline (>25 mm radius from the antibiotic disk), 30 samples showed some resistance development (<25 mm radius), 11 samples showed complete resistance (0mm radius) and 2 samples were indeterminate. This indicates a high level of Oxytetracycline resistance development in *P. larvae* in Saskatchewan.

Approximately fifteen percent of the honey samples tested was found to be indeterminate with respect to isolating *P. larvae* from contaminating species. This indicates that perhaps, changes made to the protocol could render more efficiency.

A honey screening service would be beneficial in Saskatchewan to detect American foulbrood and antibiotic-resistant bacteria. In some cases, preventative

measures could be taken for protection of hives not initially showing symptoms of American foulbrood. A honeycomb testing service could be used as a confirmation tool of the presence of American foulbrood in infected hives. The service could determine the development of antibiotic-resistant strains of *P. larvae* in specific hives and legislation could be implemented. A random screening survey throughout Saskatchewan could be conducted to determine the occurrence and special distribution of American foulbrood within the province. However, a more refined protocol may be necessary before the process is absolute and efficient.

Foulbrood Survey 2001

Sample #	Name	Result	Resistance to OTC (50 mm diam)	
F01-001	Clarence Carter	Indeterminable- unable to isolate rods from contaminatin		
F01-002	Rudi Laprell	Neg		* confirmed by BiOLOG
F01-003	Albert Robertson	Pos	No- 27 mm from dis	* confirmed by BiOLOG
F01-004	Greg Hutchings	Indeterminable- unable to isolate rods from contaminatin		
F01-005	Don Mohr	Neg		
F01-006	Pedersen Bros.	Neg		
F01-007	Tony Lalonde	Neg		* confirmed by BiOLOG
F01-008	Robert Colbert	Neg		* confirmed by BiOLOG
F01-009	Star City Colony	Indeterminable- unable to isolate rods from contaminatin		
F01-010	Knox Apiaries	Neg		* confirmed by BiOLOG
F01-011	West Cowan, Warriner	Neg		
F01-012	Otto Boettcher	Neg		* confirmed by BiOLOG
F01-013	Gerald Bayn	Pos	Yes- 0 mm from disk	* confirmed by BiOLOG
F01-014	Neil Carter	Neg		* confirmed by BiOLOG
F01-015	Neil Specht	Neg		* confirmed by BiOLOG
F01-016	Ridgedale Coop	Neg		
F01-017	Keith Groot	Neg		
F01-018	Keith Groot	Neg		*confirmed by BiOLOG
F01-019	Wink Howland	Neg		
F01-020	Dwight Sollosy	Neg		* confirmed by BiOLOG
F01-021	Larry McNaughton	Neg		* confirmed by BiOLOG
F01-022	Rick Lane 1999	Pos	Approaching- 21 mm from disk	
F01-023	Rick Lane 2000	Neg		* confirmed by BiOLOG
F01-024(5)	Neil Righi	Neg		
F01-024(12)	Neil Righi	Neg		
F01-025(435)	Tim Wendell	Neg		
F01-025(735)	Tim Wendell	Pos	Appraoching- 14 m	* confirmed by BiOLOG
F01-025(810)	Tim Wendell	Neg		* confirmed by BiOLOG
F01-025(795)	Tim Wendell	Indeterminable- unable to isolate rods from contaminatin		
F01-025(943)	Tim Wendell	Neg		
F01-025(335)	Tim Wendell	Indeterminable- unable to isolate rods from contaminatin		
F01-025(1000)	Tim Wendell	Neg		
F01-025(465)	Tim Wendell	Neg		
F01-025(750)	Tim Wendell	Neg		
F01-025(985)	Tim Wendell	Neg		
F01-026	Gerald Hiron	Indeterminable- unable to isolate rods from contaminatin		
F01-027	Richard Stushnoff	Indeterminable- unable to isolate rods from contaminatin		
F01-028	S. Gruszka	Neg		
F01-029	S2 Honey	Neg		
F01-030	Frank Colbert	Pos	Approaching- 23 mm from disk	
F01-031	Hannigan Honey	Indeterminable- unable to isolate rods from contaminatin		
F01-032	Dennis Morgan	Neg		
F01-033	Garnet Siemens	Neg		
F01-034	Carl Meyer	Neg		
F01-035	Terry Petriew	Pos	No	* confirmed by BiOLOG
F01-036	Harold & Joan Wiebe	Neg		
F01-037	Callum Honey (Bill Chalmers)	Indeterminable- unable to isolate rods from contaminatin		

F01-038	Phil Bray	Neg		
F01-039	Lester Martens	Indeterminable- unable to isolate rods from contaminatin		
F01-040	Mike Devine	Neg		
F01-041	Mike Devine	Neg		
F01-042	Mike Devine	Neg		* confirmed by BiOLOG
F01-043	Mark Wiatr	Pos	Approaching- 5 mm	* confirmed by BiOLOG
F01-044	Michael Kotelko	Neg		
F01-045	Len Proctor	Indeterminable- unable to isolate rods from contaminatin		
F01-046	Paul Antonichuk	Neg		
F01-047	Andy Raczyinski	Neg		* confirmed by BiOLOG
F01-048	Anthony Walmsley	Neg		
F01-049	John Polson	Pos	Yes- 0 mm from disk	* confirmed by BiOLOG
F01-050	Ray Bishop "R"	Indeterminable- unable to isolate rods from contaminatin		
F01-051	Ray Bishop "M"	Indeterminable- unable to isolate rods from contaminatin		
F01-052	Carolyn McDonald	Neg		
F01-053	Moyen	Neg		
F01-054	Brian Perry	Neg		* confirmed by BiOLOG
F01-055	Bill Nash	Neg		
F01-056	Harvey Boettcher	Neg		
F01-057	John Marko	Neg		
F01-058	Charles Gane	Neg		
F01-059	Charles Gane	Neg		
F01-060	James Hood	Neg		
F01-061	Richard Gane	Neg		
F01-062	Terry Allchurch	Neg		
F01-063	Blaine McKee	Neg		
F01-064	Blaine McKee	Neg		
F01-065	Calvin Parsons	Neg		
F01-066	Harvey Victor	Neg		
F01-067(1)	Yves Garez	Neg		
F01-067(39)	Yves Garez	Neg		
F01-067(49)	Yves Garez	Neg		
F01-068(3)	Jim Rehaluk	Neg		
F01-068(5)	Jim Rehaluk	Indeterminable- unable to isolat		* BiOLOG had no ID
F01-069	Brian Strong	Neg		
F01-070	Jim Hawrishok	Indeterminable- unable to isolate rods from contaminatin		
F01-071	Dwayne Tolofson	Neg		
F01-072	Cam Ferguson	Neg		
F01-073	Jim Riou	Indeterminable- unable to isolate rods from contaminatin		
F01-074	Sig Splett	Pos	Approaching- 12 mm from disk	
F01-075	John Sochaski	Neg		
F01-076	*no such sample*	N/A		
F01-077	Sarah Comerford	Indeterminable- unable to isolate rods from contaminatin		
F01-078	Don Greig	Neg		
F01-079	Ferne Porter	Neg		
F01-080	Ernie Whelpton	Pos	Yes- 0 mm from disk	* confirmed by BiOLOG
F01-081	Dennis McFarlen	Neg		
F01-082	Blackmore	Neg		
F01-083	Alvey Halbgewachs	Neg		
F01-084	Robin Lendvoy	Neg		
F01-085	Fred Lawford	Neg		

F01-086	Andy Kuderewko	Neg		* confirmed by BiOLOG
F01-087	Garnet Kummick	Neg		
F01-088	Susan Miller	Indeterminable- unable to isolate rods from contaminatin		
F01-089	Otto Gudehus	Neg		
F01-090	Bob Krause	Neg		
F01-091	Mark Grismer	Pos	Yes- 0 mm from disk	
F01-092	Norm Knudsen	Indeterminable- unable to isolate rods from contaminatin		
F01-093	Gordon Zosel	Neg		
F01-094	Bacon Apiaries	Neg		
F01-095	Wendel's Apiaries	Neg		
F01-096	Guran Honey Farm	Indeterminable- unable to isolate rods from contaminatin		
F01-097	M. McPhail	Indeterminable- unable to isolate rods from contaminatin		
F01-098	Lyle Padget	Pos	Yes- 0 mm from disk	*confirmed by BiOLOG
F01-099(1-20)	Eldon Janzen	Neg		
F01-099(24)	Eldon Janzen	Neg		
F01-099(25)	Eldon Janzen	Neg		* confirmed by BiOLOG
F01-099(26)	Eldon Janzen	Pos	Approaching- 11 mm+ from disk	
F01-100	Evelyn & Dennis Keays	Pos	Yes- 0 mm from disk	* confirmed by BiOLOG
F01-101	Hamilton Bee Ranch Ltd.	Neg		* confirmed by BiOLOG
F01-102	Emde Apiaries	Neg		
F01-103	Thickwood Honey	Indeterminable- unable to isolate rods from contaminatin		
F01-104	*none*	Neg		
F01-105	Andres Apiaries	Neg		
F01-106	Sawchyn	Pos	Approaching- 15 mm from disk	
F01-107(65)	Pipestone Apiaries	Neg		
F01-107(75)	Pipestone Apiaries	Neg		
F01-107(80)	Pipestone Apiaries	Neg		* confirmed by BiOLOG
F01-107(100)	Pipestone Apiaries	Neg		
F01-108	Walter Pylypchuk	Indeterminable- unable to isolate rods from contaminatin		
F01-109	Tony Slager	Pos	Approaching- 5 mm from disk	
F01-110	Glory Bee Honey	Neg		
F01-111	Bob Bee Honey Farm	Neg		
F01-112	Carolyn McRae	Neg		
F01-113	Ian Eaton	Neg		* confirmed by BiOLOG
F01-114	Smycniuk Honey Farms	Indeterminable- unable to isolate rods from contaminatin		
F01-115	Jason Feeley	Indeterminable- unable to isolate rods from contaminatin		
F01-116(44)	Howard Janzen	Neg		
F01-116(45)	Howard Janzen	Neg		* confirmed by BiOLOG
F01-116(49)	Howard Janzen	Indeterminable- unable to isolat		* BiOLOG had no ID
F01-117	George Knudson	Neg		* confirmed by BiOLOG
F01-118	Anderson, B & J	Neg		

Honeycomb Samples

F01-119	Polson	Pos	Approaching- 9 mm from disk	
F01-120	Marshall	Pos	Approaching- 16 m	* confirmed by BiOLOG
F01-121	Leggott	Pos	Approaching- 18 mm from disk	
F01-122	Lisa Byers	Pos	Approaching- 16 mm from disk	
F01-123	Noreen Emde	Pos	Approaching- 15 mm from disk	
F01-124	Hannigan	Pos	Indeterminable- unable to isolate rods from c	
F01-125	Mike Devine	Pos	Approaching- 18 mm from disk	

F01-126	Kirk Siebert	Pos	Approaching- 16 mm from disk
F01-127	Grant Tait	Pos	Approaching- 9 mm from disk
F01-128	Boschman	Pos	Indeterminable- unable to isolate rods from c
F01-129	Brian Strong	Pos	Approaching- 12 mm from disk
F01-130	John Reynolds	Neg	
F01-131	Fred Cronk	Pos	Approaching- 12 mm from disk
F01-132	Petriew	Pos	Approaching- 14 mm from disk
F01-133	E. Michelenko	Pos	Yes- 0 mm from disk
F01-134	Richard Bunce	Pos	Approaching- 15 mm from disk
F01-135	Larry Munn	Pos	Appraoching- 10 mm from disk
F01-136	K. Groot	Pos	Yes- 0 mm from disk
F01-137	Brian Perry	Pos	Appraoching- 22 m * confirmed by BiOLOG
F01-138	A. Slager	Pos	Yes- 0 mm from disk * confirmed by BiOLOG
F01-139	Lee Monteith	Pos	Approaching- 2 mm * confirmed by BiOLOG
F01-140	Bob Cole	Neg	
F01-141	Pedersen	Pos	Yes- 0 mm from disk
F01-142	M. Bishop	Pos	Approaching- 14 m * confirmed by BiOLOG
F01-143	Peter McKeller	Pos	Approaching- 17 mm from disk
F01-144	Bill Zacharias	Neg	
F01-145	Rodney Barber	Pos	Approaching- 2 mm from disk
F01-146	Jamie Erb	Pos	Approaching- 7 mm from disk
F01-147	Chris Warriner	Pos	Yes- 0 mm from disk
F01-148	Harry E.	Pos	Approaching- 17 mm from disk
F01-149	Otto Boettcher	Pos	Approaching- 11 mm from disk
F01-150	Alvey Halbgewachs	Pos	Indeterminable- unable to isolate rods
F01-150-honey	Alvey Halbgewachs	Pos	Approaching- 22 mm from disk

Evaluation

Positive		46
	Resistant 0 mm	11
	Approaching <25 mm radius	30
	Susceptible >25 mm radius	2
	Indeterminable	3
Negative		99
Indeterminable		26

Total 171